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Critical Illness-Related Bone Loss Is Associated With Osteoclastic and Angiogenic Abnormalities

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ABSTRACT

Critically ill patients are at increased risk of fractures during rehabilitation, and can experience impaired healing of traumatic and surgical bone fractures. In addition, markers of bone resorption are markedly increased in critically ill patients, while markers of bone formation are decreased. In the current study, we have directly investigated the effect of critical illness on bone metabolism and repair. In a human in vitro model of critical illness, Fluorescence-activated cell sorting (FACS) analysis revealed an increase in circulating CD14+/CD11b+ osteoclast precursors in critically ill patient peripheral blood compared to healthy controls. In addition, the formation of osteoclasts was increased in patient peripheral blood mononuclear cell (PBMC) cultures compared to healthy controls, both in the presence and absence of osteoclastogenic factors receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). Culturing PBMCs with 10% critically ill patient serum further increased osteoclast formation and activity in patient PBMCs only, and neutralization studies revealed that immunoglobulin G (IgG) antibody signaling through the immunoreceptor Fc receptor common γ chain III (FcRγIII) played an important role. When analyzing bone formation, no differences in osteogenic differentiation were observed using human periosteal-derived cells (hPDCs) treated with patient serum in vitro, but a decrease in the expression of vascular endothelial growth factor receptor 1 (VEGF-R1) suggested impaired vascularization. This was confirmed using serum-treated hPDCs implanted onto calcium phosphate scaffolds in a murine in vivo model of bone formation, where decreased vascularization and increased osteoclast activity led to a decrease in bone formation in scaffolds with patient serum-treated hPDCs. Together, these findings may help to define novel therapeutic targets to prevent bone loss and optimize fracture healing in critically ill patients. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: CRITICAL ILLNESS; PERIPHERAL BLOOD MONONUCLEAR CELLS; OSTEOCLASTS; FcRγIII; HUMAN PERIOSTEAL-DERIVED CELLS

Introduction

Critical illness, whether evoked by sepsis, trauma, or extensive surgery, predisposes patients to prolonged dependency on vital organ support. We have previously reported that markers of lean tissue wasting such as skeletal muscle and bone evoke the catabolic state of prolonged critical illness.[1] Specifically, circulating biomarkers of bone resorption are substantially elevated whereas markers of bone formation are low.[2] Such an imbalance may predispose critically ill patients to impaired fracture healing, osteoporosis, and increased risk of new fractures during intensive care unit (ICU) stay or rehabilitation. A recent retrospective case-cohort study revealed a significant increase in fracture risk in survivors of critical illness,[3] implying a clinically relevant impact of the reported alterations in bone biomarkers. Until now, however, no studies have directly addressed the effect of critical illness on bone metabolism and repair at the tissue and cellular level.

Normal bone turnover depends on a tight coupling between the function of mature osteoclasts, osteoblasts, and vascularization. This coupling requires a complex equilibrium of mechanical, endocrine, and nutritional factors. Prolonged critically ill patients are immobilized and suffer from a wide variety of endocrine and inflammatory disturbances, including hypercortisolism, hyposomatotropism, secondary hypothyroidism, hypogonadism, vitamin D deficiency, and elevated inflammatory cytokine levels.[2] Each of these factors, separately or in combination, may contribute to increased bone resorption and decreased bone formation.

In other disease states characterized by excessive bone loss, such as postmenopausal osteoporosis, cystic fibrosis, and Paget's disease, increased osteoclast formation and activity from peripheral blood mononuclear cells (PBMCs) has been reported,[4] both in the presence and absence of the canonical osteoclast activation factors, receptor activator of NF-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF).[5]

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Increased osteoclast formation in such disorders appears to be related to elevated serum tumor necrosis factor α (TNF-α), interleukin 1 (IL-1), and IL-6 levels[6]; however, recently, immunomodulatory factors such as Fc receptor common γ chain III (FcRγIII) have also been shown to influence osteoclastogenesis.[7] To date, the behavior of PBMCs derived from critically ill patients has not been investigated.

New bone formation and fracture healing requires precursor cells located in the periosteum, a specialized connective tissue forming a thin fibrous membrane firmly anchored to bone. Human periosteal-derived cells (hPDCs) have previously been used as a clinically relevant model to examine bone formation and fracture healing.[8]

Here, we performed a series of studies to address the impact of prolonged critical illness on bone catabolism and repair at the cellular level. In an in vitro model, we investigated whether PBMCs isolated from critically ill patients are predisposed to differentiate into osteoclasts, and the effect of humoral and immunomodulatory factors present in patient serum on osteoclast formation and activity. In addition, we assessed how these humoral factors of critical illness influenced osteoblast differentiation of human periosteal cells in vitro and bone formation in vivo in a murine model.

Patients and Methods

In vitro model of bone resorption during critical illness

Experimental subjects

We collected human peripheral blood from prolonged critically ill patients ($n = 12$, 26–80 years of age, mean age 57 ± 16.39 years) and healthy control volunteers, matched for age, sex, and body mass index (BMI) ($n = 12$, 23–81 years of age, mean age 57 ± 17.44 years) (Table 1). Serum samples were also collected from these individuals, and filtered through a 0.2-μm membrane (Millipore, Overijse, Belgium). The baseline characteristics of critically ill patients are described in Table 1. All protocols were approved by the Institutional Review Board of Leuven University. Written informed consent was obtained from all healthy volunteers and from the patients or, when the patient was unable to give consent, from the closest family member. Prior to sample collection, it was ensured that no steroidal drugs or bisphosphonates had been taken by patients or healthy volunteers in the past 12 months.

Flow cytometry

We carried out fluorescence-activated cell sorting (FACS) analysis on freshly isolated peripheral blood samples from a separate population of age-, sex-, and BMI-matched critically ill patients and healthy controls ($n = 5$ per group). Osteoclast precursors were detected by staining fresh blood samples with allophycocyanin (APC)-conjugated anti-VNR (R&D Systems, Abindgdon, UK), phycoerythrin (PE)-conjugated anti-CD14, and fluorescein isothiocyanate (FITC)-conjugated anti-CD11b (both from BD Biosciences, Erembodegem, Belgium), or with the corresponding isotype control at 4°C for 30 minutes. Following incubation with the required antibody combinations, red blood cells were lysed (RBC lysis buffer; eBioscience, Vienna, Austria), and the remaining cells washed in FACS buffer before being incubated with 7-AAD viability dye for 15 minutes. FACS analysis was then carried out immediately. We treated double-positive $CD14^+/CD11b^+$ cells as early osteoclast precursors, and triple-positive $CD14^+/CD11b^+/VNR^+$ cells as osteoclast precursors, according to the literature.[9,10] Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences). The expression of membrane antigens was analyzed using BD FACSDiva software (BD Biosciences).

Table 1. Characteristics of Critically Ill Patients and Age-, Sex-, and BMI-Matched Healthy Volunteer Controls Whose Peripheral Blood Was Used to Isolate PBMCs for In Vitro Osteoclast Differentiation Studies

Characteristic	Patients	Controls	*p*
n	12	12	
Age (years), median (IQR)	57 (26–80)	57 (23–81)	>0.99
Male gender, %	71.4	71.4	>0.99
BMI (kg/m^2), mean ± SD	24.5 ± 3.8	25.9 ± 3.5	0.37
ICU stay upon day of sampling (days), median (IQR)	13 (7–134)	NA	
APACHE-II score on admission, median (IQR)	28 (16–40)	NA	
Admission diagnosis, *n*		NA	
Complicated cardiac/vascular surgery	3		
Abdominal sepsis	3		
Burn injury	1		
Complicated neurosurgery	3		
Trauma	1		
Complicated laryngectomy	1		

APACHE II = Acute Physiology and Chronic Health Evaluation II; BMI = body mass index; ICU = intensive care unit; IQR = interquartile range; PBMC = peripheral blood mononuclear cell.

PBMC isolation and culture

We isolated PBMCs from 20 mL whole blood of patients or healthy volunteers by means of Ficoll-Paque Plus (GE Healthcare, Brussels, Belgium) density gradient centrifugation according to the manufacturer's instructions. Cells were stored in liquid nitrogen until use and seeded at a density of 5×10^5 cells per well.[11] All cultures were performed in quadruplicate in 16-well culture slides (VWR, Leuven, Belgium) or 16-well BD Biocoat Osteologic Slides (BD Biosciences), and cells were cultured in α modified essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin ("complete medium"), or complete medium plus M-CSF (25 ng/mL; R&D Systems) and RANKL (30 ng/mL; Cell Sciences, Canton, MA, USA). In order to study the effect of circulating factors on osteoclast formation, PBMCs were also

grown in complete medium containing 10% human healthy volunteer serum (HS) or 10% patient serum (PS). For cytokine and immunoreceptor neutralization experiments, PBMCs were grown in complete medium/10% PS for 14 days, in the presence of anti-TNF-α/anti-IL-6 (1 μg/mL and 8 μg/mL, respectively; R&D Systems), anti-FcγRIII (20 μg/mL), or a combination of anti-TNF-α/anti-IL-6 and anti-FcγRIII antibodies. All cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Osteoclast formation and activity

After 14 days, we fixed and stained PBMCs for the osteoclast-specific marker tartrate-resistant acid phosphate (TRAP), and the nuclear marker 4,6-diamidino-2-phenylindole (DAPI), as previously described.[4] The formation of TRAP positive multinucleated (more than three nuclei) cells was quantified by counting the multinuclear stained cells in each well.[4] Filamentous (F)-actin rings representing active osteoclasts were visualized after staining of the actin cytoskeleton with the toxin phalloidin conjugated to FITC (Sigma–Aldrich, Bornem, Belgium), and quantified by counting. To evaluate osteoclast activity, cells were removed from the hydroxyapatite-coated wells with 14% sodium hypochlorite, and the mineral layer was stained with Von-Kossa as previously described.[12] Lacunar resorption was determined by measuring the total area unstained by Von-Kossa (total area resorbed) using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and was expressed as the total percentage of the surface reabsorbed.

In-vitro model of osteoblast differentiation during critical illness

Cell culture

We obtained hPDCs from the Laboratory for Skeletal Development and Joint Disorders, Katholieke Universiteit Leuven, Leuven, Belgium, and maintained them as previously described.[13] All experiments were carried out with expanded cell populations between passage five and seven, with a seeding density of 4500 cells/cm^2. After 48 hours in culture, the growth medium of in vitro osteogenic assays was replaced using osteogenic medium, which consisted of FBS-free growth medium supplemented with 100 nM dexamethasone (Sigma-Aldrich), 10 mM glycerol-2-phosphate disodium salt hydrate (Sigma-Aldrich), 50 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich), and 10% serum (10% FBS, 10% PS, or 10% HS). The medium was replaced every 3 days.

In vitro mineralization assay

We analyzed hPDCs for in vitro mineralization using Alizarin red to detect mineral deposits, as previously described.[14] Quantification of calcium mineral deposits was performed by dissolving the dye with 10% cetylpyridinium chloride (in demineralized water) for 10 min at room temperature (RT). Absorbance was measured spectrophotometrically at 570 nm.

Gene expression analysis

We seeded hPDCs at 4500 cells/cm^2 and treated them for 7 days with osteogenic medium and FBS, HS, or PS. Total RNA was isolated using the RNeasy kit (Qiagen Benelux, Venlo, Netherlands) and cDNA was synthesized with the SuperScript III First Strand synthesis system for real-time PCR (Invitrogen). Quantitative real-time SYBR Green (Invitrogen) PCR was performed according to the manufacturer's protocol, with mRNA levels normalized to β-actin expression. SYBR Green qPCR primers were designed to span an intron so that only RNA-specific amplification was possible (runt-related transcription factor 2 [RUNX2]-F, 5′-CGCATTCCTCATCCCAGTAT-3′; RUNX2-R, 5′-GCCTGGGGTCTGTAATCTGA-3′; collagen, type I, alpha 1 [COL1A1]-F, 5′-GACGAAGACATCCCACCAAT-3′; COL1A1-R, 5′-AGATCACGTCATCGCACAAC-3′; alkaline phosphatase [ALP]-F, 5′-GGACATGCAGTACGTAGCTGA-3′; ALP-R, 5′-GTCAATTCTGCCTCCTTCCA-3′; vascular endothelial growth factor A [VEGFA]-F, 5′-CCCACTGAGGAGTCCAACAT-3′; VEGFA-R, 5′-GCATTCACATTTGTTGTGCTG-3′; vascular endothelial growth factor receptor 1 [VEGF-R1]-F, 5′-AAGCAAACCACACTGGCTTC-3′; and VEGF-R1-R, 5′-CGGGGATTTCACTGTACATCT-3′). Total RNA samples subjected to cDNA synthesis reactions in the absence of reverse transcriptase were included as negative controls and relative differences in expression were calculated using the $2^{\Delta CT}$ method.[15]

Western immunoblotting

For protein expression analysis, we seeded hPDCs at 4500 cells/cm^2 and treated them for 7 days with osteogenic medium and FBS, HS, or PS. To detect VEGFR1, Rabbit anti-human VEGFR1 primary antibody (Abcam, Cambridge, UK; 1:500) was incubated overnight at 4°C. Binding of goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP) secondary antibody (DakoCytomation, Glostrup, Denmark; 1:2000) was visualized by enhanced chemiluminescence (ECL). Normalization for total protein was performed by re-probing the membrane with mouse anti-mouse beta-actin (Abcam; 1:1000) for 1 hour at RT, followed by goat anti-mouse IgG HRP (DakoCytomation; 1:2000) for 1 hour at RT.

In vivo model of bone formation during critical illness

In vivo bone formation

We analyzed in vivo bone formation as described.[8] Briefly, hPDCs (passage five) were seeded at 4500 cells/cm^2 and treated for 7 days with DMEM and 10% FBS, HS, or PS. After 7 days, the cells were trypsin-released, centrifuged, and resuspended at a concentration of 50 million cells/mL. Subsequently, 20 μL of the cell suspension was applied to the upper surface of each 21-mm^3 calcium phosphate (CaP) NuOss scaffold. To allow cell attachment, the seeded scaffolds were incubated overnight at 37°C. After incubation, the constructs were directly implanted subcutaneously in the back at the cervical region of NMRI-nu/nu mice. The implants were collected after 56 days of implantation. Each explant was fixed in 4% formaldehyde, scanned by micro–computed tomography (μCT), decalcified in EDTA/PBS (pH 7.5) for 2 weeks, paraffin-embedded, and processed for histology. All procedures on animal experiments were approved by the local ethical committee for Animal Research (Katholieke Universiteit Leuven). The animals were housed according to the guidelines of the Animalium Leuven (Katholieke Universiteit Leuven).

Bone quantification

To quantify ectopic bone formation, we used μCT to quantify the volume of new bone formed in three dimensions by segmenting the newly formed mineralized tissues from the calcium phosphate grains in each material. For segmentation, a manually selected, but consistent global threshold value was used for each scaffold. The choice of threshold value was confirmed by visual comparison to the corresponding histological sections.

Histological analysis

We performed histological staining on paraffin-embedded sections of cell/biomaterial constructs. Hematoxylin and eosin (H&E) and TRAP staining was carried out as previously described,[8] and TRAP-positive osteoclasts were visualized using fluorescent microscopy, and quantified using ImageJ software. For CD31 immunohistochemistry, the tissue sections underwent antigen retrieval in proteinase K for 15 minutes at 95°C and were blocked in 1% normal rabbit serum, before being incubated overnight at 4°C with rat anti-mouse CD31 (BD Pharmingen; 1:20). Sections were then probed with the biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Peterborough, UK; 1:100), and the signal was amplified with the Vector Laboratories "ABC system." The signal was detected using 3,3′-diaminobenzidine (DAB), the sections were counterstained with hematoxylin, and the number of CD31-positive vessels per scaffold was visually quantified.

Statistical analysis

The data were processed using the Statistical software package StatView 5.0.1 (SAS Institute, Inc., Cary, NC, USA). Normally distributed data were analyzed using one-way ANOVA tests with a post hoc Fisher's least significant difference test for multiple comparisons. The nonparametric Kruskal-Wallis and Mann-Whitney U test were used when data appeared to be not normally distributed. No other corrections for multiple comparisons were made. Values of p less than or equal to 0.05 were considered statistically significant. Statistical significance is indicated on all graphs as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

Results

Circulating early osteoclast precursors are increased in critically ill patients

To establish whether cells from critically ill patients are triggered to differentiate into osteoclasts before any in vitro manipulation, the number of osteoclast precursors present in critically ill patient or healthy control peripheral blood was assessed by FACS analysis. Double-positive $CD14^+/CD11b^+$ cells are considered to be early osteoclast precursors, and triple-positive $CD14^+/CD11b^+/VNR^+$ cells are considered to be mature circulating osteoclasts.[4,16] The population of viable mononuclear cells were first gated for 7-AAD (viability dye) and CD14 (Fig. 1*A*). From this population, the cells that were also CD11b-positive were then gated, and classed as "early osteoclast" precursors. There were significantly more early osteoclast precursors ($CD14^+CD11b^+$) in the blood of critically ill patients than in blood from healthy controls (99.1% versus 83.9%, respectively; $p < 0.05$; Fig. 1*A*, *B*). From this population of viable, CD14/CD11b-positive cells, "late osteoclast" precursors that were also positive for vitronectin receptor (VNR) were then determined; however, no significant increase in mature circulating osteoclasts was observed ($CD14^+CD11b^+VNR^+$) (Fig. 1*A*, *C*).

Osteoclast formation is increased in critically ill patient PBMC cultures in vitro

To further examine the cause of bone loss during critical illness in a clinically relevant model, PBMCs were isolated and pooled from an age-, sex-, and BMI-matched set of critically ill patients and healthy controls, and analyzed for osteoclast formation and activity. To assess whether these precursor cells form active osteoclasts, the isolated PBMCs were cultured for 14 days in FBS, with and without RANKL and M-CSF, and the number and activity of osteoclasts in patient cultures were compared with healthy matched controls. After 14 days of culture with RANKL and M-CSF, the formation of mature, multinuclear ($\geq$3 nuclei, TRAP-positive) osteoclasts was 17-fold higher in PBMC cultures from critically ill patients than those from healthy controls (Fig. 2*A* (I), *B*; $p < 0.05$). Analysis of osteoclast activity was carried out by quantifying the degree of hydroxyapatite resorption after culturing the cells for 21 days in hydroxyapatite-coated wells.[12] This revealed a 36-fold higher resorption in patient PBMC cultures with RANKL and M-CSF as compared with controls (Fig. 2*A* (II), *C*; $p < 0.05$). Importantly, PBMCs from critically ill patients also differentiated into osteoclasts without RANKL and M-CSF (24-fold increase versus healthy cells); however, these osteoclasts were unable to resorb hydroxyapatite, suggesting that the presence of humoral osteoclastogenic factors is necessary for the formation of active osteoclasts (Fig. 2*A* (II), *C*).

Autologous critically ill patient serum increases osteoclast formation and activity

To assess the impact of humoral factors in the patient's blood on the differentiation potential of PBMCs into osteoclasts, PBMCs from patients and healthy controls were cultured in the presence of 10% autologous patient serum (PS) or 10% autologous healthy serum (HS). Culturing patient PBMCs in 10% PS resulted in a 4-fold increase in osteoclast formation compared to patient PBMCs grown with 10% HS, both with and without RANKL and M-CSF (Fig. 3*A*, *B*; $p < 0.05$). Importantly, osteoclasts formed from patient PBMCs in PS without RANKL and M-CSF also displayed increased F-actin ring formation (6-fold), and actively resorbed hydroxyapatite (2-fold increase) compared to patient cultures grown in 10% HS (Fig. 3*A*, *C*, *D*; $p < 0.05$). These data suggest the presence of humoral factors present in patient serum that promote mature, active osteoclast formation and activity.

Interestingly, the addition of 10% PS to cultures of healthy control PBMCs did not increase osteoclast formation or resorption, in the presence or absence of RANKL and M-CSF (Fig. 3*A*–*C*). These data suggest a unique interaction between patient cells and PS that is not present in healthy control cultures and PS.

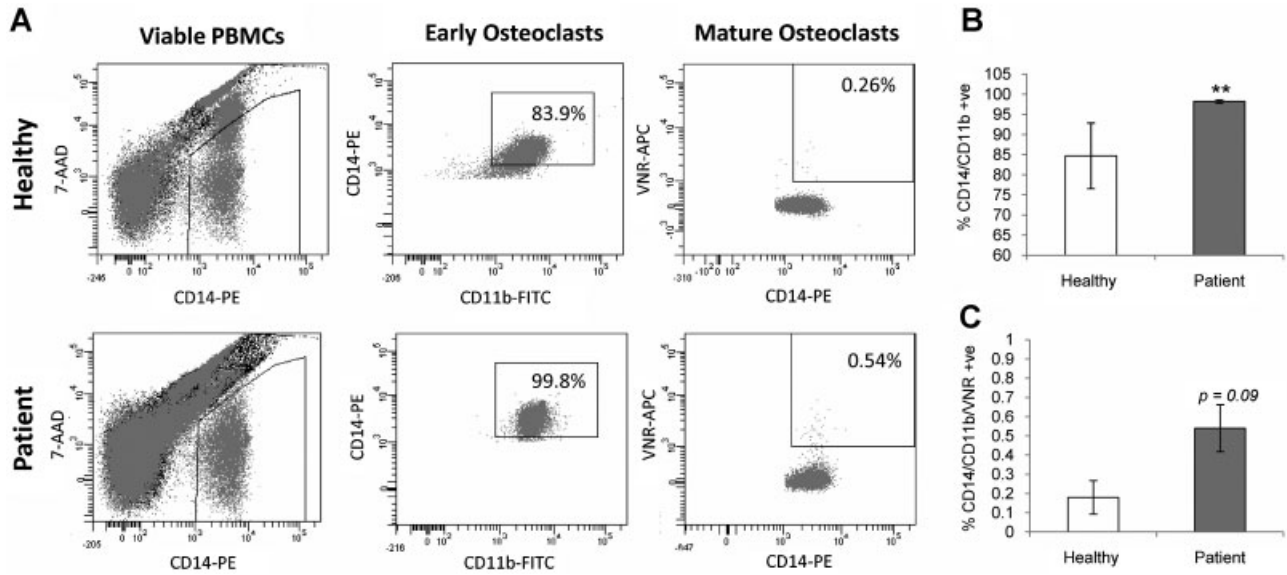

Fig. 1. Circulating osteoclast precursors in healthy and critically ill patient peripheral blood. (*A*) Representative dot plots of the gating of viable $CD14^+$ PBMCs, $CD14^+/CD11b^+$ early osteoclast precursors and $CD14^+/CD11b^{+/}VNR^+$ mature osteoclasts in healthy and patient peripheral blood samples matched for age, sex, and BMI. (*B*) FACS analysis revealed a significant increase in early osteoclast precursors ($CD14^+/CD11b^+$) from the peripheral blood of critically ill patients (99.8% ± 0.38% and 83.9% ± 7.09%, respectively) ($n = 5$; $^{**}p < 0.01$). (*C*) A trend toward an increase in mature circulating osteoclasts ($CD14^+/CD11b^{+/}VNR^+$) was also observed ($n = 5$; $p = 0.09$).

Inhibition of the immunomodulatory receptor FcγRIII in patient serum decreases in vitro osteoclast formation in patient PBMCs

In order to assess whether the humoral factors that seem to interact with the primed PBMCs from critically ill patients could be attributed to the elevated levels of the major proinflammatory cytokines or other immunomodulatory factors, cells were grown in 10% PS with neutralizing antibodies for inflammatory cytokines IL-6 and TNF-α, and/or the immunoreceptor FcγRIII. Interestingly, blocking IL-6 or TNF-α activity did not reduce the formation of TRAP- and F-actin ring–positive, actively resorbing osteoclasts (Fig. 4*A*–*C*). However, upon the addition of anti-FcγRIII, osteoclast formation and activity was significantly reduced compared to control cultures (69.2%; $p < 0.05$), or compared to PBMCs grown with cytokine neutralizing antibodies (61.4%; $p < 0.05$) (Fig. 4*A*–*D*).

Critically ill patient serum does not affect osteoblast differentiation but reduces expression of angiogenesis markers in vitro

In order to assess the impact of circulating factors during critical illness on bone formation, an in vitro model of osteogenesis during critical illness was established. For this purpose, we used hPDCs, a population of mesenchymal cells isolated from the human periosteum, that have shown to proliferate, migrate, and differentiate into chondrogenic and osteogenic lineages upon stimuli such as trauma, fracture, or infection.[13,17] Cells were cultured for 21 days in osteogenic medium, as previously described.[13,18] As a late marker of hPDC osteoblast differentiation, calcium deposition was measured quantitatively using Alizarin Red staining on differentiated hPDCs.

Addition of FBS, HS, or PS to the osteogenic medium did not affect differentiation or mineralization of the cells (Fig. 5*A*, *B*). Also, gene expression analysis of osteogenic markers RUNX2 and COL1A1 (type I collagen) revealed no difference between PS and HS (despite a reduction in expression of these genes in both these groups as compared with cells grown in FBS) (Fig. 5*C*, *D*). Expression of ALP (bone-specific alkaline phosphatase) was also not different between PS and HS (Fig. 5*E*). These data suggest that osteogenic differentiation is unaffected by circulating factors during critical illness assessed in vitro. Interestingly, expression of the angiogenesis factor VEGF alpha (VEGFA) was not different between PS and HS groups (Fig. 5*F*), although a reduction in VEGF-receptor 1 (VEGFR1) expression with PS as compared with HS was detected at the gene and protein level, suggesting that angiogenesis may be compromised during bone formation and skeletal healing in critical illness (Fig. 5*G*, *H*).

In an in vivo murine model, bone formation is reduced with patient serum, coinciding with increased osteoclast activity and reduced angiogenesis

Bone formation is a multifactorial process involving a tightly regulated cascade of events, which include the recruitment, proliferation, and differentiation of osteoprogenitor cells, along with the formation of a well-defined vascular compartment. Therefore, although no differences in osteogenesis in the presence of PS were observed with hPDCs in vitro, it was plausible that aberrant bone formation might still occur in vivo, possibly through deficient angiogenesis. In order to test this hypothesis, hPDCs were incubated with FBS, HS, or PS for 7 days in basal medium, before being seeded onto calcium phosphate

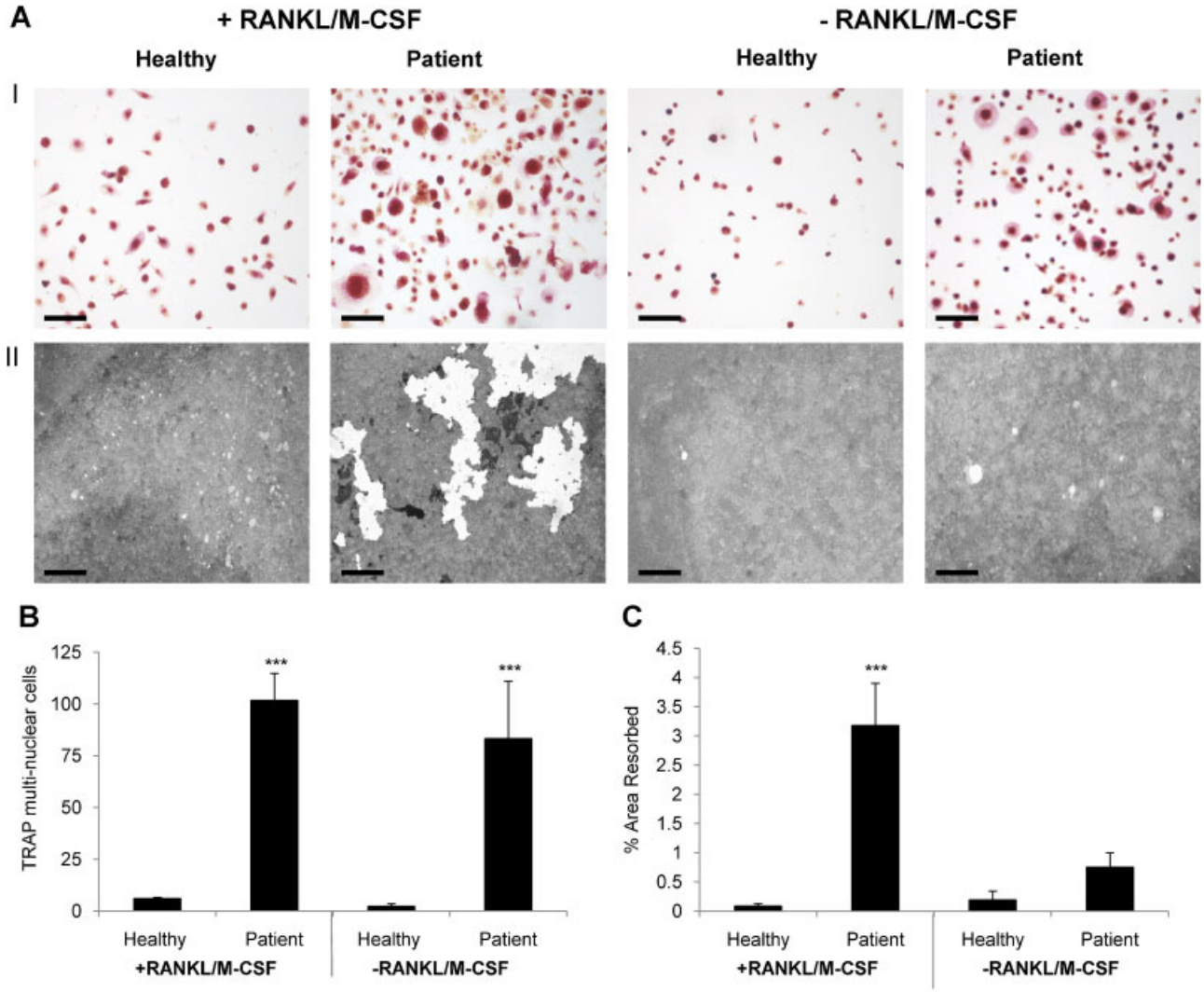

Fig. 2. Human in vitro study of osteoclast differentiation. (*A*) Formation of mature, multinuclear osteoclasts was visualized by TRAP positivity (scale bar = 100 μm) (I) and resorption on hydroxyapatite (scale bar = 500 μm) (II) in healthy or patient cultures with FBS, in the presence and absence of RANKL and M-CSF. (*B*) The number of TRAP-positive multinuclear cells was significantly increased in critically ill patient PBMC cultures with and without RANKL and M-CSF. (*C*) Resorption of hydroxyapatite was increased in patient cultures in the presence and absence of RANKL and M-CSF ($n = 8$; $^{***}p < 0.05$ versus healthy cells).

(CaP) NuOss scaffolds overnight, transplanted into NMRI-nu/nu mice, and incubated for 8 weeks.[8] Upon explantation and analysis of bone formation by μCT, scaffolds containing PS-treated hPDCs revealed significantly less mature bone than those containing HS-treated cells (28.9% reduction) (Fig. 6*A*). No significant differences in calcium phosphate granules or fibrous tissue compartments were observed. In view of the increased osteoclast activity observed in previous experiments, possibly contributing to bone hyperresorption in critical illness, the trend for an increase in osteoclastic activity in PS scaffolds, as detected by TRAP staining, was unsurprising (Fig. 6*B*). The local vasculature is vital for the formation of new bone during normal bone maintenance and following fracture. Upon explant, PS scaffolds contained visibly less vascularization than HS scaffolds. Therefore, the formation of blood vessels was measured with CD31 immunohistochemistry, which revealed that vascularization was significantly reduced in PS scaffolds (Fig. 6*C*).

Discussion

In the current study we have attempted to identify the cellular and humoral factors regulating bone resorption and formation during critical illness. The cellular effects of human critical illness were studied in vitro, where PBMC osteoclast precursors in the blood of critically ill patients seemed to possess a long-lasting "memory" of the critical illness, resulting in increased osteoclast formation compared to healthy controls. This was further potentiated by humoral factors present in the serum from critically ill patients. Unexpectedly, neutralizing the inflammatory cytokines TNF-α and IL-6 in patient serum did not inhibit osteoclast formation in patient PBMC cultures; however, blocking signaling through the immunoreceptor FcRγIII inhibited osteoclast formation both in the presence and absence of cytokine neutralizing antibodies, suggesting immunomodulatory mechanisms may be involved. In an in vitro model of bone

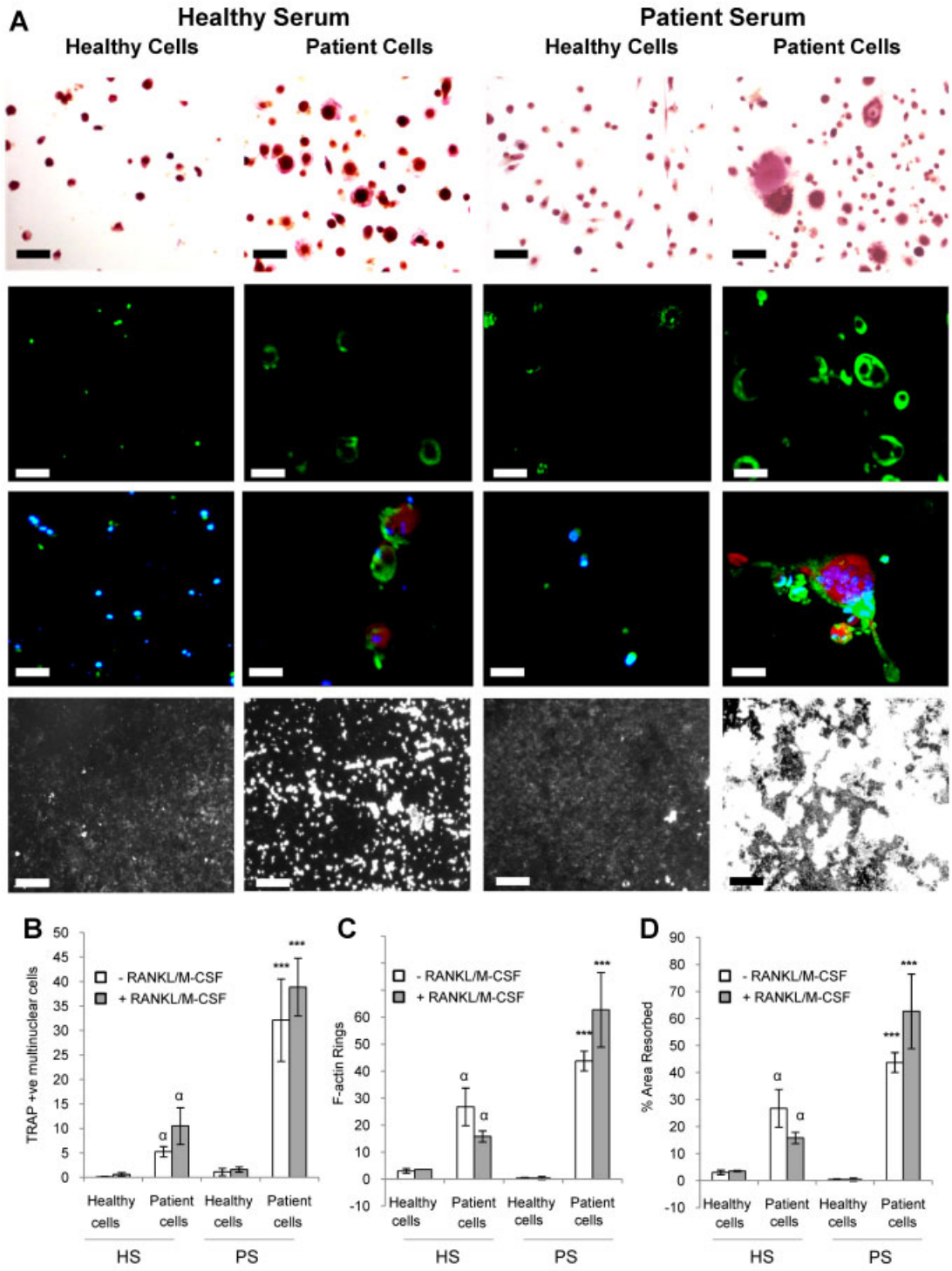

Fig. 3. Effect of critically ill patient serum on osteoclast differentiation and activity. (*A*) Formation of mature, multinuclear osteoclasts was visualized by TRAP positivity (top row), F-actin ring formation (second row), formation of multinuclear cells (third row, merged image of TRAP, DAPI, and F-actin ring staining) (scale bar = 100 μm), and resorption on hydroxyapatite (bottom row; analyzed with Von Kossa staining; scale bar = 500 μm) in pooled PBMC cultures from healthy controls and critically ill patients. (*B*) The number of TRAP-positive multinuclear cells was significantly increased in patient PBMC cultures without (white bars) and with (gray bars) osteoclastogenic factors RANKL and M-CSF, in the presence of PS. (*C*) F-actin ring formation was also significantly increased in the presence of patient serum in patient PBMC cultures in the absence (white bars) and presence (gray bars) of RANKL and MCSF. (*D*) Resorption of hydroxyapatite was increased in patient cultures cultured with 10% HS or 10% PS in the absence (white bars) and presence (gray bars) of RANKL and MCSF ($n = 8$; $^{***}p < 0.05$ versus patient cells plus HS and healthy cells plus PS; $^{\alpha}p < 0.05$ versus healthy cells plus HS).

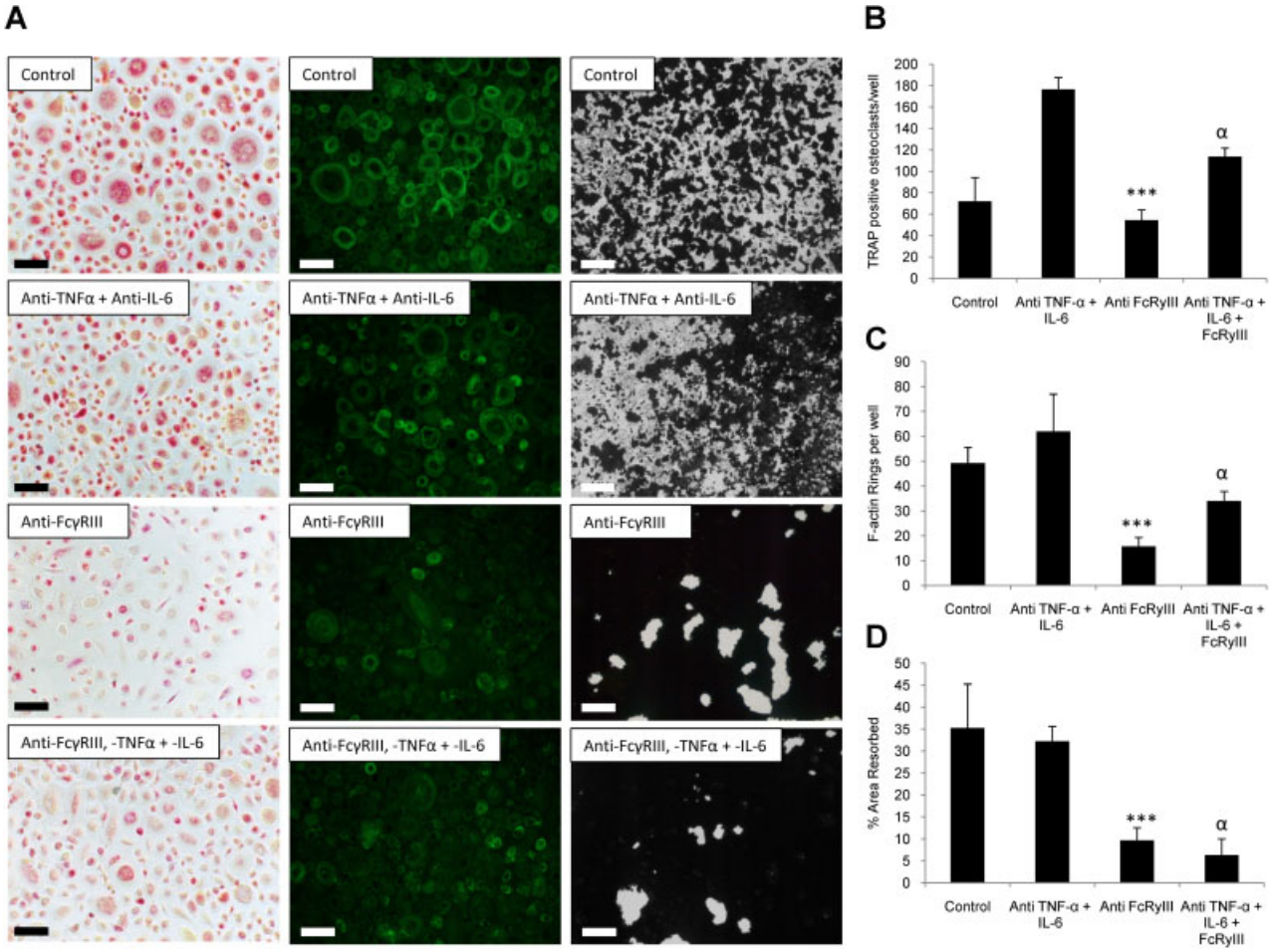

Fig. 4. Inhibition of the immunomodulatory receptor FcγRIII in patient serum decreases in vitro osteoclast formation. (*A*) Formation of mature, multinuclear osteoclasts was visualized by TRAP (left panel; scale bar = 100 μm), F-actin ring formation (middle panel; scale bar = 100 μm), and resorption (right panel; scale bar = 100 μm) in pooled PBMC cultures from critically ill patients with 10% PS and anti-IL-6/TNF-α (1 μg/mL and 8 μg/mL, respectively), anti-FcγRIII (20 μg/mL), or a combination of anti-IL-6/TNF-α and anti-FcγRIII. The number of TRAP-positive multinuclear cells (*B*), F-actin rings per well (*C*), and the percentage area resorbed (*D*) was significantly reduced in patient PBMC cultures with anti-FcγRIII, or a combination of anti-FcγRIII and anti-IL-6/TNF-α, compared to control cultures, or cultures with anti-IL-6/TNF-α alone ($n = 4$; $^{***}p < 0.001$ versus control cultures, $^{\alpha}p < 0.001$ versus anti-IL-6/TNF-α).

formation, we were unable to reveal an effect of critical illness on osteoblast differentiation that could explain the reduction of bone formation observed in vivo, although a reduction in VEGF-R1 gene and protein expression in vitro, together with a reduction in vascularization in vivo suggests that reduced angiogenesis may play a role.

During critical illness, markers of bone resorption are highly elevated by up to 4-fold, whereas markers of bone formation are reduced.[2] In addition, survivors of critical illness experience an increased risk in fragility fractures up to 8 years following ICU discharge.[3] Although critically ill patients are immobilized for long periods of time, in a recent study analyzing bone turnover markers in 40 patients immobilized by stroke for a minimum of 6 months, the bone resorption marker Serum CrossLaps was increased by 2-fold,[19] suggesting that the extreme bone hyperresorption observed during critical illness cannot be explained by immobilization alone.

In our study, FACS analysis carried out on fresh human peripheral blood samples from critically ill patients revealed a significant increase in circulating early osteoclast precursors, suggesting that, even prior to in vitro manipulation, critically ill patient PBMCs display increased osteoclastogenic potential. This increase in osteoclast precursors is often characterized by an increase in osteoclast formation and activity, which can be recapitulated in vitro through the isolation and differentiation of PBMCs into osteoclasts, with the addition of osteoclastogenic factors such as RANKL and M-CSF. In the current study, PBMCs were isolated from healthy controls and critically ill patients and pooled, in order to reduce genetic variability and study the effect critical illness on osteoclast formation, rather than individual patient conditions. Pooled PBMCs from critically ill patients displayed a significant increase in differentiation into osteoclasts compared to healthy controls, both in the presence and absence of RANKL and M-CSF. However, these mature, multinuclear osteoclasts were only able to actively resorb hydroxyapatite in the presence of humoral factors. Numerous other studies have reported that, in vitro, PBMCs isolated from patients of Paget's disease, osteoporosis, phenylketonuria, multiple myeloma, and

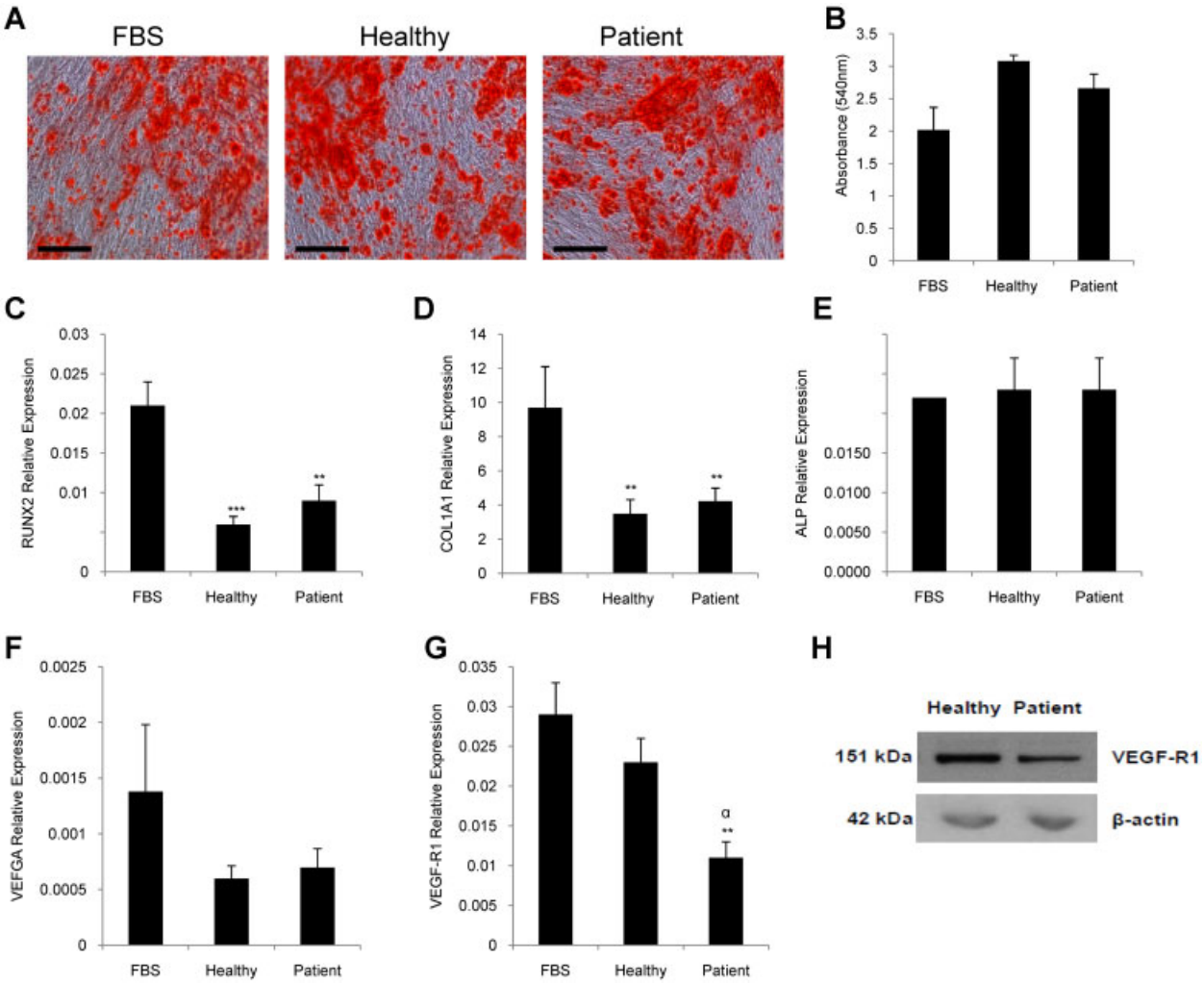

Fig. 5. Effect of critically ill patient serum on osteogenic differentiation and angiogenesis in vitro. (*A*) Alizarin Red staining of hPDC monolayers cultured for 21 days in 10% FBS, 10% HS ("Healthy") or 10% PS ("Patient"). (*B*) Alizarin Red staining quantified by assessment of optical density at 540 nm revealed no significant difference in mineralization between Healthy and Patient serum treatment. qRT-PCR analysis of (*C*) RUNX2, (*D*) COL1A1, (*E*) ALP, and (*F*) VEGFA normalized to β-actin revealed no differences in gene expression between HS and PS conditions, although a reduction in VEGF-R1 expression was observed in patient serum conditions (*G*), which was also observed at the protein level (*H*). ($n = 8$; $^{***}p < 0.001$ versus FBS; $^{**}p < 0.01$ versus FBS; $^{\alpha}p < 0.01$ versus healthy serum).

rheumatoid arthritis display spontaneous differentiation into mature osteoclasts.[4,9,16,20] Interestingly, during in vitro osteoclast differentiation of PBMCs from chronic liver disease patients with osteopenia,[21] "spontaneously" formed osteoclasts required the exogenous addition of M-CSF and RANKL to resorb bone, suggesting that these factors are essential for osteoclast activity.

In the current study, osteoclast formation and activity was further increased in patient PBMC cultures upon the addition of 10% PS, whereas no increase was observed with HS, or in healthy PBMC cultures treated with PS. This suggested that crosstalk between factors within the PS and patient PBMCs may have promoted osteoclastogenesis. Inflammatory cytokines have previously been shown to promote the formation of osteoclasts in vitro and in vivo,[22–24] and have been directly related to fragility fractures in postmenopausal females.[22] However, surprisingly, neutralizing antibodies for both TNF-α and IL-6 did not suppress osteoclast formation in patient PBMC cultures with 10% PS, suggesting that other factors present in the serum such as RANKL may have been involved. RANKL and osteoprotegerin (OPG) have both been previously measured in patients with critical illness.[2] Although levels of RANKL were undetectable, levels of OPG were significantly increased 3-fold in critically ill patients compared to healthy controls upon intensive care admission, and decreased significantly throughout the time in intensive care (while levels of serum β–cross-linked C-telopeptide [CTX] and urinary deoxypyridinoline [DPD] increased). High serum concentrations of the decoy receptor OPG and low serum concentrations of RANKL have been reported in disorders resulting in secondary osteoporosis, such as primary biliary cirrhosis.[25] In addition, a number of studies have reported increased levels of serum OPG in multiple myeloma (MM) patients compared to healthy controls, which decreases over time.[7] Recently, the importance of immunomodulatory factors in diseases of bone metabolism has become apparent.

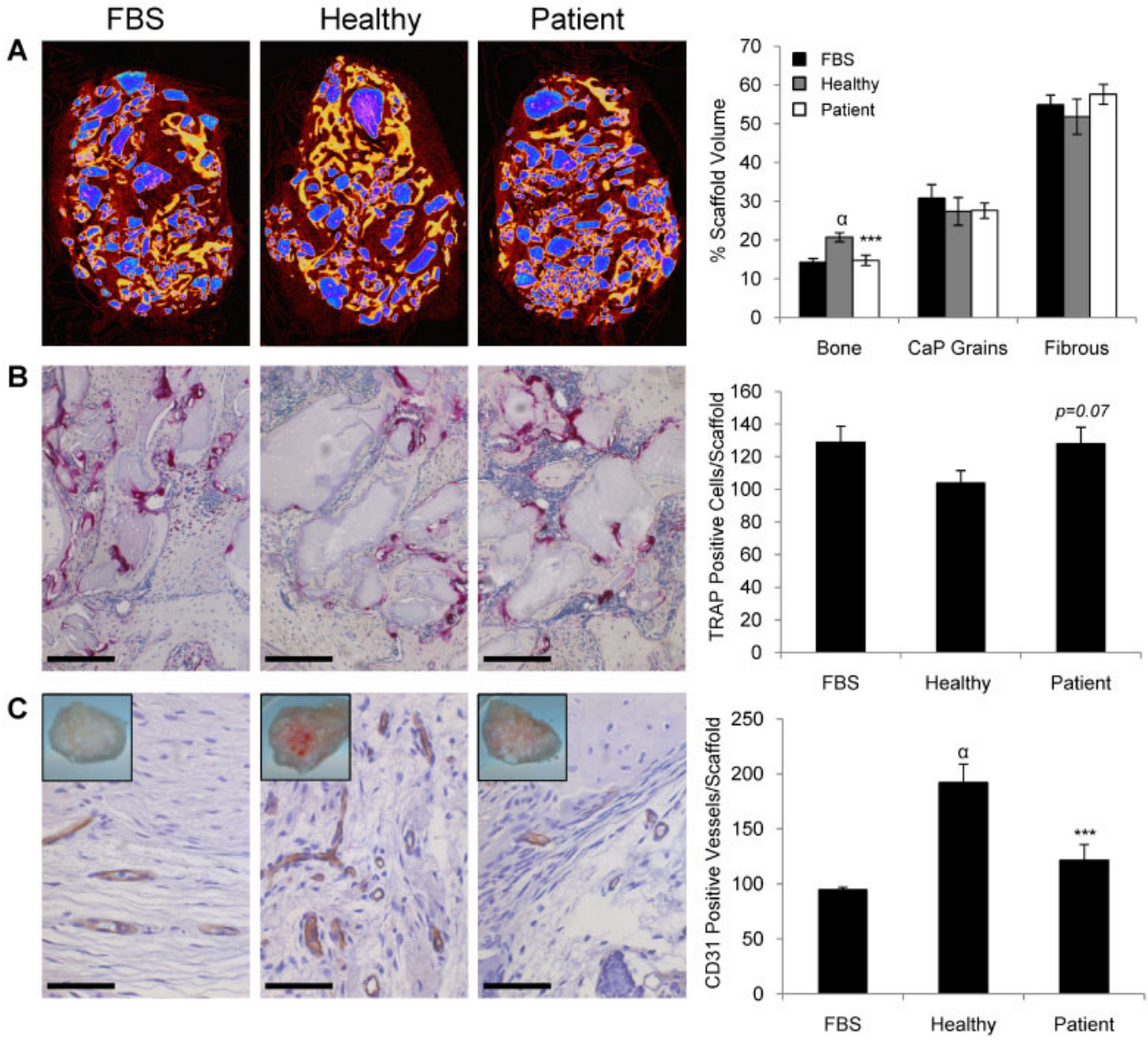

Fig. 6. Effect of critically ill patient serum on bone formation in vivo. (*A*) Bone quantification of hPDC NuOSS implants was carried out using μCT analysis 8 weeks after implantation in nude mice, and revealed a significant reduction in bone formation in patient-serum conditions compared to healthy serum (mature bone = yellow; remaining CaP grains = blue). No significant differences in CaP grains or fibrous tissue were detected in the scaffold. (black bars = FBS; gray bars = healthy serum; white bars = patient serum). (*B*) A trend toward an increase in the number of TRAP-positive osteoclasts per scaffold in patient serum-treated hPDCs was observed, although this did not reach significance ($p = 0.07$; scale bar = 200 μm). (*C*) Upon explant, scaffolds coated with patient serum-treated hPDCs had visibly less vasculature than scaffolds coated with healthy serum hPDCs (insets). This was confirmed by quantification of CD31 immunohistochemistry, with a significant reduction in the number of CD31-positive blood vessels in scaffolds with patient serum treated hPDCs (scale bar = 50 μm) ($n = 4$; $^{***}p < 0.001$ versus healthy serum; $^{\alpha}p < 0.01$ versus FBS).

In immune complex–mediated reactions, the amplitude of inflammatory responses is believed to depend on the ratio of activating and inhibitory Fcγ receptors (FcγR). Three classes of FcγR are expressed in monocytes: FcγRI, FcγRII, and FcγRIII. FcγRIII (also known as CD16) is an activating receptor whose expression is increased in inflammatory bone disorders such as psoriatic arthritis, rheumatoid arthritis, and osteoporosis.[7,20] In the current study, blocking FcγRIII/CD16 expression in patient PBMC cultures caused a significant decrease in osteoclast formation, which was sustained when inflammatory cytokines IL-6 and TNF-α were also blocked. This suggests that humoral factors and/or IgG antibodies in critically ill patient serum may be acting through the FcγRIII/CD16 receptor to increase osteoclast formation in patient PBMC cultures.

In addition to an increased risk of fractures during rehabilitation, such bone hyperresorption could also predispose critically ill patients to impaired healing of traumatic and surgical bone lesions. For instance, prolonged critical illness may compromise sternal healing following sternotomy for cardiac surgery, resulting in poor outcome.[26] In order to investigate the effect of critical illness on bone formation during skeletal repair, critically ill patient serum was applied to the cells presently considered essential for fracture healing, namely hPDCs.[8,17,27]

In order to heal the fracture, multipotent periosteal cells must differentiate into osteoblasts to mediate bone formation. Previous studies have reported that human serum isolated from juvenile idiopathic arthritis patients has a negative effect on osteoblast differentiation in vitro, possibly due to the high levels of cytokines present in the serum.[28] Therefore, the impact of prolonged critically ill patient serum on the differentiation capacity of hPDCs was evaluated by mineralization of the extracellular matrix with calcium deposition. Although an increase in mineralization was observed in all differentiated conditions compared to nondifferentiated cells, no differences were observed between HS or PS. Similarly, gene expression of RUNX2, COL1A1, and ALP revealed no differences in expression between HS and PS conditions, although a reduction in RUNX2 and COL1A1 expression was observed in both human serums compared to the standard FBS condition. Although these findings suggest that in vitro, factors such as inflammatory cytokines present in critically ill PS do not have an effect on osteogenic differentiation, the fact that the expression of VEGF-R1 at both the gene and protein level was significantly reduced with PS suggests that vascularization may be inhibited during skeletal healing and bone formation in critical illness.

This hypothesis was corroborated by the in vivo model of bone formation during critical illness, where a reduction in bone formation in PS-coated NuOss scaffolds implanted in NMRI-nu/nu mice correlated with a significant reduction in vascularization, along with an increase in osteoclast activity. This finding directly supports the hypothesis that hypoxia is a major risk factor for impaired fracture healing, such as evidenced by sternal healing problems after internal mammary artery harvesting for coronary bypass surgery.

The present study has, for the first time, investigated the difficult problem of extreme bone loss during critical illness at the cellular and tissue level. Increased circulating osteoclast precursors in critically ill patients seemed to maintain their osteoclastogenic phenotype when studied in vitro, and were stimulated further by factors such as IgG antibodies present in critically ill patient serum, which enhanced the "primed" osteoclast activity. Additionally, although osteogenesis did not seem to be affected by critical illness in vitro, bone formation in calcium phosphate scaffolds was hampered in vivo, possibly due to reduced angiogenesis brought about by circulating factors. The identity of these factors remains to be investigated.

Our data may help to define novel therapeutic targets to prevent bone loss and optimize fracture healing in critically ill patients.

Disclosures

All authors state that they have no conflicts of interest.

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